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Influence of 2(3-Methyl-cinnamyl-hydrazono)-propionate on Glucose and Palmitate Oxidation in Human Mononuclear Leukocytes

Hydrazonopropionic Acids, a New Class of Hypoglycaemic Substances, VII[1])

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Summary: 2-(3-Methyl-cinnamyl-hydrazono)-propionate stimulated glucose oxidation in human mononuclear leukocytes and the stimulation was similar to that by concanavalin A. Both substances must affect glucose metabolism at two sites, the first site being before the pyruvate dehydrogenase step because of the increase of lactate plus pyruvate concentration. The second site is related to pyruvate oxidation.

The hydrazone inhibited the conversion of palmitate to CO_2. This effect could have caused an activation of the pyruvate dehydrogenase complex, resulting from a decreased acetyl-CoA/CoA ratio. Concanavalin A did not influence fatty acid oxidation. Both substances did not affect the CO_2 formation from acetate.

Mononuclear leukocytes appear to be a suitable model for the investigation of the influence of hypoglycaemic substances on glucose and fatty acid metabolism in living human cells.

Introduction

Hydrazonopropionic acids have been found to lower the blood glucose concentration in various laboratory animals (1–3). 2-(3-Methyl-cinnamyl-hydrazono)-propionate (MCHP) is considered to be most promising as a potential oral antidiabetic substance among various derivatives of hydrazono-propionate tested so far (4, 5).

The mechanism of action appears to be multifactorial because hepatic gluconeogenesis and jejunal glucose uptake are influenced by these compounds. Recently we have found that the glucose consumption by hemidiaphragms of rats is stimulated by 2-(3-methyl-cinnamyl-hydrazono)-propionate (6). However, all experiments have so far been performed in animal models.

Human mononuclear blood cells have become increasingly useful for the study of insulin receptors (7–10) and various metabolic pathways, especially in glucose and fatty acid metabolism (11–13). The entire *Embden-Meyerhof* pathway and the complete oxidation of fatty acids including the citric acid cycle operate in lymphocytes (14, 15). Many inborn errors of glucose and fatty acid metabolism have also been detected in these blood cells (16–18). HL 60 leukaemic cell lines have been shown to be unable to grow in an insulin-free medium (19). The raised level of activated T lymphocytes precedes the earliest abnormalities of glucose tolerance in insulin-dependent diabetes (20). Hence, it appears reasonable to study the action of pharmacologically effective substances on these pathways in mononuclear leukocytes. They represent easily accessible living cells which can be obtained from healthy and diseased persons, thus avoiding the use of laboratory animals.

[1]) Part VI see l. c. (30).

We have therefore investigated whether glucose consumption is also influenced by 2-(3-methyl-cinnamyl-hydrazono)-propionate in human mononuclear leukocytes.

Since lymphocytes have been widely used to study the stimulation of glucose oxidation by concanavalin A (21, 22), this compound was included in the present experiments for purposes of comparison.

Materials and Methods

Materials

Sodium metrizoate-Ficoll (Lymphoprep, cat. No. 350031, density 1.077) was purchased from Molter (D-6900 Heidelberg), Heparin Novo (5000 IU · ml^{-1}) from Novo Industry Pharmazeutika (D-6500 Mainz).

2-(3-Methyl-cinnamyl-hydrazono)-propionate was generously provided by Prof. Dr. *F. H. Schmidt* and Dr. *H. Kühnle* (Boehringer Mannheim GmbH, D-6800 Mannheim). On the day before the experiment 2-(3-methyl-cinnamyl-hydrazono)-propionate was freshly dissolved in 100 ml *Krebs-Ringer* bicarbonate solution (pH = 7.4) and treated with ultrasound for about 30 minutes to achieve a clear solution.

All enzymes, coenzymes and fatty acid-free bovine albumin (cat. No. 775 835) were purchased from Boehringer Mannheim GmbH (D-6800 Mannheim), Hionic Fluor (cat. No. 6013319) from Packard Instruments International S. A. (CH-Zürich).

Concanavalin A from Serva AG (D-6900 Heidelberg, cat. No. 27648, 102 000) and all other chemicals were from E. Merck AG (D-6100 Darmstadt).

Labelled compounds were obtained from Amersham Buchler (D-3300 Braunschweig): sodium [1-^{14}C]acetate (code No. CFA. 13; 2.18 GBq · $mmol^{-1}$), sodium *D, L*-[1-^{14}C]lactate (code CFA. 89; 2.0 GBq · $mmol^{-1}$), [1-^{14}C]pyruvic acid (code CFA. 703; 381 MBq · $mmol^{-1}$), *D*-[U-^{14}C]glucose (code CFA. 96; 10.4 GBq · $mmol^{-1}$). [1-^{14}C]Palmitic acid (code No. CFA. 23; 2.15 GBq · $mmol^{-1}$) was diluted 1 : 50 in *Krebs-Ringer* bicarbonate solution (pH 7.4) containing 39 g · l^{-1} albumin and mixed one hour at 37 °C with a magnetic stirrer.

Preparation of mononuclear leukocytes

Mononuclear leukocytes were isolated according to *Fink* et al. (23) on a Lymphoprep density gradient from about 80 ml freshly drawn venous blood (20 ml plastic syringes were rinsed with Heparin Novo). The mononuclear leukocyte fraction contained on average about 96% lymphocytes and 4% monocytes. The blood was taken from healthy members of the hospital staff at 7.30 a.m. after their usual breakfast. The haemogram (performed with a Technicon H 6000 analyser), blood glucose and HbA_1 concentration were in the reference range. The cell fraction was washed twice with phosphate-buffered sodium chloride solution (13.7 mmol/l, pH 7.2) containing 2 g · l^{-1} bovine albumin and then suspended in 10 ml *Krebs-Ringer* bicarbonate solution containing 2 g · l^{-1} albumin. The cells were counted with a Sysmex cc-700 analyser according to the manufacturers' instructions (TOA Medical Electronics Co, D-2000 Hamburg). The cells were stained with trypan blue; as a sign of viability more than 98% of cells excluded the dye.

Mononuclear leukocyte suspension (700 µl, containing about 3×10^6 cells) were incubated in a 50 ml Erlenmeyer flask at 37 °C (shaking cycles 134 min^{-1}) with 700 µl *Krebs-Ringer* bicarbonate solution containing 2 or 20 g · l^{-1} bovine albumin (pH = 7.4), 50 µl substrate solution and 50 µl labelled substrate solution. The experiments were started by adding the leukocyte suspension. Then the flasks were closed with a rubber cap. The cap carried a glass container for the later addition of sodium hydroxide.

$^{14}CO_2$ collection

After incubation of the leukocyte fraction with labelled substrates for 60 or 180 minutes, the experiment was terminated by injecting 0.5 ml perchloric acid (0.6 mol · l^{-1}) and 0.5 ml NaOH (1 mol · l^{-1}) through the rubber cap and continuing the incubation for one hour. The glass containers carrying the NaOH were then transferred from the rubber cap into the scintillation cocktail (10.7 ml Hionic Fluor) and counted in a Beckman LB 1801 liquid scintillation counter (Beckman Instruments, D-8000 Munich). Water condensed at the outer surface of the glass container was carefully wiped off.

Determination of metabolite concentrations

For the determination of metabolite concentrations the reaction mixture was centrifuged (10 min, 5000 *g*) after the addition of 0.5 ml perchloric acid (0.6 mol · l^{-1}); the supernatant was neutralised with K_2CO_3. Glucose and glycogen were determined with the method of *Keppler & Decker* (24), pyruvate according to *Lamprecht & Heinz* (25) and lactate with the monotest from Boehringer Mannheim (cat. No. 149 993).

Statistical evaluation

The mean values are given with their standard deviations. The means are compared with each other by the *Wilcoxon* signed rank test or paired t-test. Differences are considered to be significant if $p \leq 0.05$. The number of experiments was related to the number of cell preparations from various individuals.

Results

The formation of CO_2 from [U-^{14}C]glucose was stimulated by 2-(3-methyl-cinnamyl-hydrazono)-propionate in human mononuclear leukocytes. The maximal effect was obtained with a concentration of 0.2 mmol/l (fig. 1). At higher concentrations the activation declined. The stimulatory effect of 2-(3-methyl-cinnamyl-hydrazono)-propionate (tab. 1) was reduced in the presence of higher glucose (10 mmol/l) and albumin concentrations (20 g/l).

The mononuclear leukocytes (10^{10} cells) consumed 447 µmoles glucosyl units in 3 hours. This rate was elevated in the presence of 2-(3-methyl-cinnamyl-hydrazono)-propionate to 792 µmoles (tab. 2). The pyruvate production was distinctly increased and the lactate formation not significantly affected by 2-(3-methyl-cinnamyl-hydrazono)-propionate (tab. 2). Therefore, the lactate/pyruvate ratio decreased in the perchloric acid extract of the incubation medium from 15 to 2 in the presence of 2-(3-methyl-cinnamyl-hydrazono)-propionate.

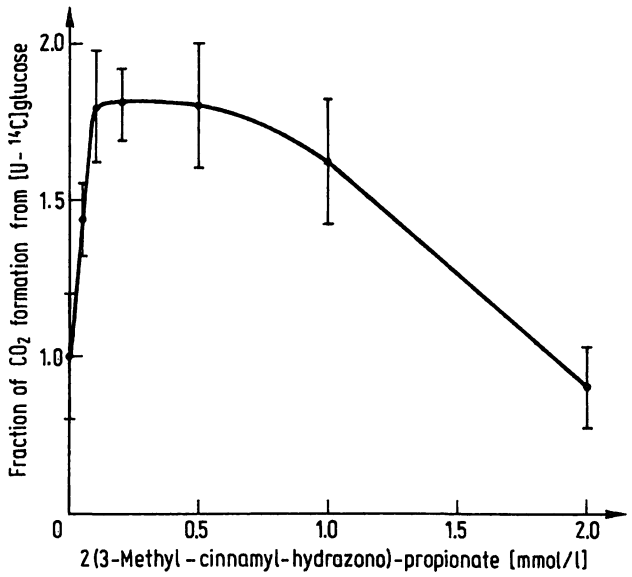

Fig. 1. The influence of 2-(3-methyl-cinnamyl-hydrazono)-propionate on CO_2 formation from glucose in human lymphocytes. Incubation time: 3 hours.
$\bar{x} = \bar{x} \pm s$, n = 5.

The ^{14}C transfer from uniformly labelled glucose to CO_2 was also stimulated by concanavalin A (tab. 2). When 2-(3-methyl-cinnamyl-hydrazono)-propionate and concanavalin A were added together, each in a concentration causing maximal stimulation, additive effects on the consumption of glucosyl units were not observed (tab. 2). In contrast to 2-(3-methyl-cinnamyl-hydrazono)-propionate, the addition of concanavalin A caused an increase of lactate, leading to an approximate 20-fold increase in the lactate/pyruvate ratio (tab. 2).

In the presence of labelled pyruvate (1.0 mmol/l) or lactate (2.0 mmol/l) the CO_2 release was also stimulated by 2-(3-methyl-cinnamyl-hydrazono)-propionate and concanavalin A (tab. 3) with slight additive effects of both compounds. When labelled lactate or pyruvate were used together with unlabelled glucose (2.0 mmol/l) only 2-(3-methyl-cinnamyl-hydrazono)-propionate increased the CO_2 formation, whereas concanavalin A was without effect and even prevented the stimulation by 2-(3-methyl-cinnamyl-hydrazono)-propionate (tab. 4).

Tab. 1. The influence of 2-(3-methyl-cinnamyl-hydrazono)-propionate (MCHP) on the transfer of ^{14}C from [U-^{14}C]glucose into CO_2 in human mononuclear leukocytes. Figures are mean values in MBq per 10^{10} cells from n different leukocyte preparations with standard deviation. Incubation: 3 h at 37 °C.

Albumin	(g · l^{-1})	2	20	20
Glucose	(mmol · l^{-1})	<0.1	<0.1	10.0
	(kBq per assay)	24.7	24.7	123.3
$^{14}CO_2$ formation, control	(MBq/10^{10} cells)	3.95 ± 1.32	5.98 ± 3.00	0.063 ± 0.022
$^{14}CO_2$ formation, MCHP, 0.2 mmol/l	(MBq/10^{10} cells)	6.38 ± 2.18	7.13 ± 3.48	0.078 ± 0.037
	(Fraction of control)	1.62	1.20	1.23
n		10	18	6
t[1])		5.62	5.32	2.49
p		<0.0005	<0.0005	<0.05

[1]) paired t-test between control and "MCHP"-values

Tab. 2. The influence of 2-(3-methyl-cinnamyl-hydrazono)-propionate (MCHP, 0.2 mmol/l) and concanavalin A (0.65 µmol/l) on the glucose consumption and on the pyruvate, lactate and CO_2 formation of human leukocytes. The glucose concentration in the reaction mixture was 2 mmol/l ([U-^{14}C]glucose 24.6 kBq).
The figures are mean values (µmol per 10^{10} cells) from 5 experiments with standard deviation. Incubation: 3 h at 37 °C.

	Control	MCHP	Concanavalin A	MCHP + concanavalin A
Glycosyl units[1]) consumed (µmol/10^{10} cells)	447 ± 145	792 ± 180*	1353 ± 279*	1272 ± 300*
Lactate formed (µmol/10^{10} cells)	669 ± 56	717 ± 225	1446 ± 432*	1371 ± 534*
Pyruvate formed (µmol/10^{10} cells)	45 ± 15	330 ± 144*	75 ± 21	351 ± 177*
CO_2 formed (µmol/10^{10} cells)	12.9 ± 3.6	26.4 ± 5.7*	44.1 ± 6.3*	49.2 ± 12.6*

[1]) The difference between the sum of glucose and glycogen-glycosyl units at 180 minutes and at the beginning of the incubation period.
* This value differs significantly from its corresponding control value.

The oxidation of long-chain fatty acids to CO_2 as measured by the ^{14}C transfer from [1-^{14}C]labelled palmitate to CO_2 was reduced to 27% by 0.2 mmol/l 2-(3-methyl-cinnamyl-hydrazono)-propionate (tab. 1), whereas the metabolism of acetate to CO_2 was not affected (tab. 5). Concanavalin A did not influence the oxidation of palmitate nor of acetate.

Discussion

2-(3-Methyl-cinnamyl-hydrazono)-propionate and concanavalin A stimulated the conversion of glucose to CO_2 as indicated by the increased ^{14}C transfer from uniformly labelled glucose (tab. 2).

Both substances must have affected the glucose metabolism at least at two sites: one is probably related to the pyruvate dehydrogenase complex and the other site is before this step. Since the ^{14}C transfer was also stimulated from [1-^{14}C]pyruvate as well as from [l-^{14}C]lactate it can be assumed that the hydrazonopropionate affected either pyruvate transport into the mitochondria or the pyruvate dehydrogenase complex. It is improbable that a later step of the pyruvate metabolism was involved because the CO_2 formation from [1-^{14}C]acetate was not influenced by both compounds. This finding excludes an uncoupling effect. It has been shown that carbonylcyanide chlorophenylhydrazone (CCCP), an uncoupler of oxidative phosphorylation, stimulates the oxidation of both [1-^{14}C]pyruvate and [1-^{14}C]acetate (26).

Tab. 3. The influence of 2(3-methyl-cinnamyl-hydrazono)-propionate (MCHP, 0.2 mmol/l) and concanavalin A (0.7 µmol/l, 72 mg/l) on CO_2 formation in the presence of [1-^{14}C] pyruvate (1.5 kBq, 1.0 mmol/l) and [1-^{14}C]lactate (3.7 kBq, 2.0 mmol/l) in mononuclear leukocytes. The figures are mean values per 10^{10} cells with standard deviation. Incubation: 3 h at 37 °C.

	Pyruvate (n = 6)			Lactate (n = 7)		
	CO_2 (kBq/10^{10} cells)	CO_2 (µmol/10^{10} cells)	(Fraction of control)	CO_2 (kBq/10^{10} cells)	CO_2 (µmol/10^{10} cells)	(Fraction of control)
Control	39.0 ± 14.5	3.96 ± 1.20		16.3 ± 4.2	3.72 ± 0.99	
MCHP	48.3 ± 12.8*	4.95 ± 0.93*	1.25	35.3 ± 10.8*	8.03 ± 2.49*	2.16
Concanavalin A	73.0 ± 13.5*	6.42 ± 1.70*	1.62	53.5 ± 15.3*	12.11 ± 3.50*	3.26
MCHP + Concanavalin A	74.0 ± 21.5*	8.22 ± 1.47*	2.08	52.7 ± 17.7*	12.80 ± 3.70*	3.44

* this value differs significantly from its corresponding control value

Tab. 4. The influence of 2(3-methyl-cinnamyl-hydrazono-propionate (MCHP, 0.2 mmol) and concanavalin A (0.7 µmol/l, 72 mg/l) on the transfer of ^{14}C from [1-^{14}C]pyruvate (1.5 kBq, 3.9 nmol) and [1-^{14}C]lactate (3.7 kBq, 3.9 nmol) into CO_2 in the presence of 2.0 mmol · l^{-1} glucose by human mononuclear leukocytes. Albumin: 2 g · l^{-1}. Figures are mean values in kBq per 10^{10} cells from different cell preparations with standard deviation. Incubation: 3 h at 37 °C.

	Pyruvate CO_2 formation		n	Lactate CO_2 formation		n
	(kBq/10^{10} cells)	(Fraction of control)		(kBq/10^{10} cells)	(Fraction of control)	
Control	41.3 ± 10.2		5	39.7 ± 23.2		4
MCHP	87.5 ± 23.5*	2.12	5	66.3 ± 24.8*	1.67	4
Concanavalin A	35.2 ± 12.3	0.85	5	38.3 ± 14.3	0.97	4
Concanavalin A + MCHP	41.5 ± 22.8	1.00	5	45.3	1.14	2

* this value differs significantly from its corresponding control value

Tab. 5. The influence of 2(3-methyl-cinnamyl-hydrazono)-propionate (MCHP, 0.2 mmol/l) and concanavalin A (0.7 µmol/l, 72 mg/l) on CO_2 formation from [1-^{14}C]acetate (1.78 kBq; 1.0 mmol · l^{-1}) and [1-^{14}C] palmitate (7.4 kBq). The figures are mean value per 10^{10} cells with standard deviation. Incubation: at 37 °C.

	n	Acetate CO_2 formation		n	Palmitate CO_2 formation	
		(µmol/10^{10} cells)	(Fraction of control)		(kBq/10^{10} cells)	(Fraction of control)
Control	4	11.6 (2.3)	–	13	800 ± 317	–
MCHP	4	11.4 (1.5)	1.00	13	217 ± 100	0.27
Concanavalin A	4	14.9 (4.5)	1.28	4	733 ± 200	0.92

A direct effect of 2-(3-methyl-cinnamyl-hydrazono)-propionate on the pyruvate dehydrogenase in mitochondrial extracts from guinea pig livers has been excluded (27). The activity of the pyruvate dehydrogenase is regulated by the degree of phosphorylation of the α-subunit of the enzyme. The phosphorylated form of the enzyme is inactive while the dephosphorylated form is active. Regulation of the phosphorylation occurs through the action of a kinase and a phosphatase contained within the multienzyme complex. Activation of the pyruvate dehydrogenase is obtained if the kinase reaction is reduced, e.g. by a decrease of mitochondrial concentration ratios of NADH/NAD^+, acetyl-CoA/CoA or ATP/ADP (28, 29). All three ratios are influenced by the rate of fatty acid oxidation. If this is inhibited lower ratios can be expected.

The cytoplasmic concentration ratio of NADH/NAD^+ was reduced in human mononuclear leukocytes by 2-(3-methyl-cinnamyl-hydrazono)-propionate, as indicated by the lactate/pyruvate ratio. The hepatic acetyl-CoA/CoA ratio was found to be reduced in the presence of 2-(3-methyl-cinnamyl-hydrazono)-propionate. This suppression was explained by an inhibition of the fatty acid oxidation, which has been shown to occur in the perfused guinea pig liver (3). In human mononuclear leukocytes the oxidation of palmitate was also significantly reduced by 2-(3-methyl-cinnamyl-hydrazono)-propionate (tab. 5) and could have led to a low acetyl-CoA/CoA ratio.

The primary event in the mechanism of hypoglycaemia produced by 2-(3-methyl-cinnamyl-hydrazono)-propionate could be an inhibition of long-chain fatty acid oxidation which depends on the carnitine acylcarnitine translocase system (30). Further effects can then be interpreted as secondary phenomena: inhibition of hepatic gluconeogenesis, jejunal inhibition of glucose uptake, stimulation of glucose oxidation in the diaphragm (6) and in mononuclear leukocytes. Presumably the inhibition of gluconeogenesis is the major cause of the hypoglycaemia observed in fasted animals, whereas the other effects of 2-(3-methyl-cinnamyl-hydrazono)-propionate may be additional factors in lowering the blood glucose concentration.

The effect of 2-(3-methyl-cinnamyl-hydrazono)-propionate on *Randle*'s glucose-fatty acid cycle (31) appears to be similar to that observed with other inhibitors of fatty acid oxidation (31, 32). The glucose-fatty acid cycle also operates in mononuclear leukocytes (to be reported in a subsequent paper). The mechanism of action suggested for 2-(3-methyl-cinnamyl-hydrazono)-propionate seems to be different from that of concanavalin A because the latter substance did not influence the oxidation of palmitate.

In the presence of 2 mmol · l^{-1} unlabelled glucose, CO_2-formation from [1-^{14}C]-pyruvate was stimulated by 2-(3-methyl-cinnamyl-hydrazono)-propionate but not by concanavalin A, although the lactate concentration was significantly increased by the latter compound. No explanation can be offered for this observation.

The concentration of lactate plus pyruvate was significantly increased in the presence of 2-(3-methyl-cinnamyl-hydrazono)-propionate or concanavalin A (tab. 2). Therefore, the activation of the pyruvate oxidation as indicated by the CO_2 formation (tab. 3) cannot completely explain the effect of both compounds on the glucose metabolism. It must be assumed that the metabolic flow rate between glucose and pyruvate is also stimulated. Apparently the pyruvate formed cannot then be oxidized fast enough, and therefore tends to accumulate.

Several authors have suggested that stimulation of glycolysis by concanavalin A results from the coordinated activation of plasma-membrane glucose transport and phosphofructose kinase in thymocytes (33, 34). Furthermore, the mitogen caused a specific increase in pyruvate oxidation and a disproportionately large conversion of glucose into lactate (36). *Brand* et al. (22, 36) have observed that the ratio of $^{14}CO_2$ released from [1-^{14}C]glucose to that from [6-^{14}C]glucose is both small and similar in the absence and presence of concanavalin A, suggesting that glucose metabolism via the oxidative segment of the pentose pathway is not enhanced relative to the tricarboxylic acid cycle oxidations in mitogen-activated rat thymocytes.

Concanavalin A is a well-known stimulator of mitogenesis. The hydrazonopropionate was without any stimulatory effect up to a concentration of 1.0 mmol l^{-1} (38) using human mononuclear leukocytes in the mitogen stimulation assay (38).

In conclusion, the effect of 2-(3-methyl-cinnamyl-hydrazono)-propionate on the oxidation of glucose and palmitate in leukocytes was similar to that observed in rat hemidiaphragm. The experimental model of mononuclear leukocytes, representing more than 95% lymphocytes, appears to be well suited for investigating the effect of hypoglycaemic substances on glucose and fatty acid metabolism in human cells.

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